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(54) Title: FACTORS HAVING PRION-BINDING ACTIVITY IN SERUM AND PLASMA AND AGENTS TO DETECT TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHITIS

(57) Abstract: Disclosed are methods and tools for the concentration and detection as well as quantification of pathological prion proteins as well as agents to be used in said detection and/or in the prevention or treatment of prion diseases. Said agents are factors with prion binding activities found in blood serum and blood plasma.

FACTORS HAVING PRION-BINDING ACTIVITY IN SERUM AND PLASMA AND AGENTS TO DETECT TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHITIS

Cross Reference to Related Application

This application claims the priority of US patent application 09/407,667, filed September 28, 1999, the disclosure of which is incorporated herein by reference in its entirety.

Field of the Invention

The present invention concerns a method and agents to detect transmissible spongiform encephalopathies as well as agents for the prevention and treatment of respective infections.

Background Art

According to all available evidence, the agents causing transmissible spongiform encephalopathies, termed prions, are devoid of informational nucleic acids and consist of an "infectious" protein (termed PrP^{Sc}) capable of converting a normal host protein called PrP^{C} into a likeness of themselves. The only organ system in which histopathological damage and its clinical sequelae can be demonstrated as a consequence of infection with prions is the nervous system (Brandner et al., 1996). This consideration applies to both the human transmissible spongiform encephalopathies, such as Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker Syndrome, Kuru and fatal familial insomnia, and all known prion encephalopathies of animals (Weber and Aguzzi, 1997). The latter comprise scrapie in sheep, bovine spongiform encephalopathy, and chronic wasting diseases of mule, deer and exotic ungulates (Weissmann and Aguzzi, 1997).

However, there is no doubt that prions, herewith operationally defined as the infectious agents causing transmissible spongiform encephalopathies, can colonize organs other than the central and peripheral nervous system, and can be demonstrated in extracerebral compartments (Aguzzi et al., 1997). The problem of which organ systems can harbour infectivity is further complicated by the existence of prion strains. Just like strains of conventional viruses, prions can come in various different flavors, each one of which has its specific preferences with regard to the host range which is infectible and also to the type of cells in which it replicates (Aguzzi, 1998). One paradoxical situation, which is of immediate relevance to the question of blood safety, is exemplified by the radically different organ tropism of the BSE agent in cows and in humans. BSE prions seem to be largely confined to the neural compartment of cows, even after oral exposure (Wells et al., 1998). A very accurate study of the pathogenesis of experimental BSE in cows upon feeding 100 grams of infected brain has disclosed that there is only a short and transient period during which infectivity can be demonstrated in the terminal ileum (Wells et al., 1998). At later time points, BSE prions can only be shown in brain, spinal cord, and dorsal root ganglia. The exact localization of BSE in the terminal ileum is not known. It is being discussed whether infectivity resides in Peyer's patches or in the neural compartment which comprises the Plexus submucosus Meissner and the Plexus myentericus Auerbach. There is a great body of circumstantial evidence that BSE prions can provoke new variant Creutzfeldt-Jakob Disease (nvCJD) (Bruce et al., 1997; Chazot et al., 1996; Hill et al., 1997; Will et al., 1996), but no absolutely final evidence has been produced. For the purpose of the following discussion we will regard the evidence that BSE and new variant Creutzfeldt-Jakob Disease caused by the same agent as

sufficiently verified (Aguzzi and Weissmann, 1996). Upon passage into humans, and consecutive progression to manifest nvCJD, prions experience a dramatic shift in their organotropism. Instead of remaining confined mainly to neural structures, they can be detected in many organs belonging to the immune system, including most notably tonsils, spleen, and as recently demonstrated, the appendix (Hilton et al., 1998). It is, therefore, unavoidable to conclude that the tropism of the infectious agent for various structures depends on both the strains of prions in question (and therefore it is in part autonomous to its carrier) and on the species in which prion disease manifests itself (Aguzzi and Weissmann, 1998).

These considerations are not only of academic interest. In fact, the transmissibility of the agent by iatrogenic manipulations (i.e. blood transfusions, organ transplants, etc.) is crucially affected by such parameters.

Horizontal Transmissibility of Human Prions:

Prion diseases of humans are undoubtedly transmissible. However, transmission is achieved only under particular circumstances. One could say that in this respect prion diseases fulfill the characteristics of transmissibility delineated by Semmelweiss for puerperal fever: these affections are infectious but not contagious. Direct transmissions of brain-derived material from a patient suffering from Creutzfeldt-Jakob disease to other persons have documentedly resulted in transmission of disease. A particularly tragic case occurred in the early seventies in Zurich, when electrodes used for cortical recordings from Creutzfeldt-Jakob patients were sterilized (formaldehyde and alcohol) and used in additional patients. Disease was transmitted to the very young recipients (Bernoulli et al., 1977).

Also, transplantation of cornea has most likely resulted in transmission of disease (Duffy et al., 1974).

Despite these tragic dimensions, cases of iatrogenic transmission of CJD via neurosurgical procedures have remained rather rare. This is not totally understood, given that the frequency of subclinical CJD must be much higher than that of manifest disease, and that most neurosurgical instruments are not sterilized in a way that would reliably inactivate prions. Therefore, the quite rare nature of iatrogenic transmission is likely to indicate that host factors, in addition to the virulence of prions, may affect the probability that infection takes place. This notion is strengthened by the epidemiology of iatrogenic CJD (iCJD) upon transmission of contaminated dura mater. It has been estimated that several thousands patients, predominantly in Japan, may have been exposed to the CJD agent via preparations of cadaveric dura mater which had been contaminated with prions. However, it appears that less then 2% of those exposed have developed disease so far. While we can rejoice about this low efficiency in the "take" of infectivity, we do not fully understand the biological basis for the apparent protection enjoyed by most subjects exposed to CJD prions. The largest problem with iatrogenic transmission has occurred as result of administration of pituitary hormones of cadaveric origin (Gibbs et al., 1985). Preparations of growth hormone and of gonadotropins contaminated with human prions have caused the death of more then 80 persons, predominantly children. Due to the long latency that can be expected when the agent is introduced into extracerebral sites, such as via intramuscular injection, it must be assumed that further cases from this procedure, which has been stopped more than a decade ago, will arise in the future.

Besides its tragic human dimension and the harm that it has cost to the patients and to their physicians, the pituitary hormone disaster needs to be

understood in detail, because the anterior lobe of the pituitary gland is not a part of the central nervous system. Therefore, these events may serve as a paradigm for transmission of prions via contaminated extracerebral tissue that does not belong to the canonical sites of replication of prions. The observation that latency after intracerebral contamination is much shorter than latency after peripheral infection is in good agreement with experimental data from various animal models, and suggest that a rather lengthy phase of extracerebral events (which may include replication of the agent, and invasion of specific extraneuronal systems) may be a precondition to prion neuroinvasion (Aguzzi, 1997).

Factors influencing the neurotropism of prions:

There is good reason to suspect that neuroinvasive processes in the course of prion infections are very tightly controlled. Perhaps the best argument in this respect derives from the observation that the incubation times of experimental animals inoculated intraperitoneally with scrapie prions are extremely reproducible. Upon inoculation with a known amount of standard inoculum, the experience in various laboratories has been that latencies between inoculation and first clinical symptoms display standard variations in the order of only a few percent points (Klein et al., 1997). If prion neuroinvasion were a totally random process, one would expect a large variability in the incubation times, which would depend on processes governed by chance. However, if some rate-limiting processes control neuroinvasion, these may be responsible for the remarkable precision of the incubation times. Indeed, we very much hope that this interpretation is correct because if such processes exist they might be amenable to manipulation, which in turn may represent a post-exposure strategy to prevent overt prion disease. Indeed, various

mechanisms have been explored by which neuroinvasions may be accomplished.

A first phase or neuroinvasion seems to be widespread colonization of the immune system. This colonization can be visualized by homogenizing spleen, lymph nodes, tonsils, and also appendix, and injecting the homogenates into suitable experimental animals. The dilution of the homogenates at which 50% of the experimental animals become sick, contains one ID50 of the infectious agent in each inoculum.

The second phase of neuroinvasion seems to be dependent upon a compartment which cannot be replaced by adoptive bone marrow transfer (Blättler et al., 1997) and which may be represented by the peripheral nervous system and/or the follicular dendritic cells resistant to germinal center of secondary lymphatic organs. It appears that this second compartment necessitates the expression of normal prion protein in order to support neuroinvasion (Blättler et al., 1997).

Neuroinvasion is dependent on a functional immune system, and immunodeficient mice do not develop disease after inoculation with a moderate dose of the agent (Fraser et al., 1996; Kitamoto et al., 1991; Lasmezas et al., 1996; O'Rourke et al., 1994). One crucial component of the immune system necessary for neuroinvasion has been traced to the physical presence of terminally mature B-lymphocytes . To date, it is not clear whether B cells are required because they bind physically prions and carry them to sites of neuroinvasion, or whether B cells produce factors, or induce processes, which are indirectly responsible for facilitating neuroinvasion (Klein et al., 1997). Given the requirement for B-lymphocytes secreting lymphotoxin for the maturation of follicular dendritic cells, and the fact that follicular dendritic cells accumulate large amounts of scrapie prions in experimental situations, it is tempting to speculate that the main function of B-

lymphocytes in the aforementioned process consists in allowing FDCs to mature.

The cellular and molecular basis of prion neuroinvasion:

Following experimental inoculation of mice with prions at peripheral sites, there is typically a prolonged, clinically silent replication phase of the infectious agent within the lymphoreticular system (LRS). This occurs prior to detectable neuroinvasion by prions and the subsequent occurrence of neurological symptoms. During this preclinical latency period, prions may replicate to high titers within lymphoreticular tissues. Elucidating the cell types in which prions replicate within the peripheral lymphoid tissue and - crucially - how prions are transported to the central nervous system (CNS) is of great interest and clinical importance. Despite considerable evidence implicating the role of the immune system in peripheral prion pathogenesis, there have been few studies on the identity of the cells involved in this process. It has been shown many years ago that whole-body irradiation of mice with gamma rays fails to influence prion pathogenesis or incubation time of scrapie. This has been taken as an argument against significant involvement of proliferating cells in the lymphoreticular phase of prion propagation. Instead, follicular dendritic cells (FDC) have been considered as the prime cell type for prion replication within lymphoid tissue since PrP^{Sc} accumulates in the follicular dendritic network of scrapie infected wild-type and nude mice (Kitamoto et al., 1991). In addition, severe combined immuno deficient mice (SCID), which lack mature B- and T-cells, and which do not appear to have functional FDCs, are highly resistant to scrapie after intraperitoneal inoculation and fail to replicate prions in the spleen (Fraser et al., 1996; Kitamoto et al., 1991; Lasmezas et al., 1996; O'Rourke et al., 1994).

Interestingly, bone-marrow reconstitution of SCID mice with wild-type spleen cells restores full susceptibility to scrapie after peripheral infection (Fraser et al., 1996; Klein et al., 1998). These findings suggest that an intact, or at least partially functional, immune system comprising lymphocytes and FDC is required for efficient transfer of prions from the site of peripheral infection to the CNS.

The time course for the development of scrapie disease following intracerebral or intraperitoneal inoculation is highly reproducible and is primarily dependent on the dose of the inoculum. Therefore, neuroinvasion by prions migrating from peripheral lymphoid tissue may depend on tightly controlled, rate-limiting reactions. In order to identify such rate-limiting steps during prion neuroinvasion, PrPC deficient mice bearing PrP-overexpressing cerebral neurografts were infected intraperitoneally (i.p.). No disease was observed in the grafts, suggesting that neuroinvasion depends on PrP expression in extracerebral sites. This was further underlined by reconstitution of the lymphoid system with PrPC expressing cells, which restores infectivity in the lymphoid tissue, but still fails to transport prions to the nervous system.

As prions can be detected in lymphoreticular tissues, an understanding of the peripheral pathogenesis is of immediate importance in assessing risks of iatrogenic transmission of human BSE via exposure to blood or tissues from preclinical cases, and possibly from contaminated surgical instruments, or even blood and blood products. Additionally, such advances might pave the way for the development of sensitive diagnostic tests and the means to block prion neuroinvasion. Why is contamination of the blood supply with prions an important issue? The main problem is new variant CJD. For one thing, we by far do not know as much about the epidemiology and iatrogenic transmissibility of this new

disease as we do for sporadic CJD (sCJD). What is most unsettling, the distribution of preclinical disease in Great Britain and possibly in other countries is very obscure, and the little knowledge that is being gathered is far from reassuring (Will et al., 1999). Moreover, there is all reason to believe that nvCJD may be much more "lymphoinvasive" than its sporadic counterpart. In particular, nvCJD prions can be easily detected in lymphatic organs such as tonsils and appendix (Hill et al., 1999; Hill et al., 1997; Hilton et al., 1998), a fact that was previously demonstrated to be true for scrapie (Schreuder et al., 1997; Schreuder et al., 1998; Vankeulen et al., 1996), but not for sCJD prions. While all available evidence points to follicular dendritic cells as the prion reservoir in lymphatic organs, splenic lymphocytes of experimentally inoculated mice can be infected with prions (Raeber et al., 1999). Although prion infectivity of circulating lymphocytes appear to be at least two logs lower than that detected in splenic lymphocytes (Raeber et al., 1999), the possibility that circulating lymphocytes may be in equilibrium with their splenic siblings call for cautionary measures. The nature of the latter is still matter of controversy and debate: leukodepletion has been advocated, but at present there is no certainty about its efficacy, and even whether the presently available technologies for leukoreduction are necessary and/or sufficient for decreasing the threat to blood supply that derives from nvCJD. In addition, it has to be taken into account that, even if blood prion infectivity were to be originally contained in lymphocytes in vivo, lysis of cells may lead to contamination of non-particulate fractions and, in the absence of appropriate measures of removal, of stable blood products.

The second consideration applies so secondary prophylaxis. Given the very large numbers of infectious BSE material that has entered the human food chain, it is

possible that many individuals harbor preclinical nvCJD. It is imperative and urgent to develop strategies that will help control spread of the agent and that will hopefully prevent the clinical outbreak of symptoms in these persons. Possible targets for the interference with neuroinvasion are rate-limiting processes that control prion replication within the infected individual. In light of the knowledge discussed above, treatments that target the neuro-immune interface of prion replication and neuroinvasion (Aguzzi and Collinge, 1997) seem a promising area for research aimed at post-exposure prophylaxis.

Methods to detect prions and their limitation:

In the age of real-time kinetic polymerase chain reaction (PCR), we have become very spoiled with respect to the detection thresholds which we demand from assays geared at detecting viral contaminants in blood. Consider the case of HIV: here the introduction of quantitative PCR technologies has pushed the limit of detection in blood and blood products down to quasi-perfection. Even when PCR techniques have not proved that useful, or have not yet met with such widespread acceptance, ultrasensitive immunochemical methods, such as time-resolved fluorescent ELISA, have progressed to a degree of sophistication that is highly satisfactory for most screening application. So why do we still have a problem with prion detection in blood?

The most formidable problem derives from the unique biology of the prion. According to more-or-less accepted wisdom, infectious prions are likely to consist solely of the PrP^{Sc} protein, which has exactly the same amino acid structure as the normal cellular protein PrP^{C}. A more noncommittal way of wording this fact would be to state that PrP^{Sc} is the only known surrogate marker for prion infectivity: this latter statement is likely to be

agreeable upon by both the proponents of the protein-only hypothesis and by those who still believe that the infectious agent is a virus.

The consequence of the fact mentioned above for prion detection is obvious: if prion-specific nucleic acids do not exist, any PCR-based screening assay to detect said nucleic acid will not be an option. Therefore, we are left with immunochemical assays. Besides being less sensitive than PCR by several orders of magnitude, these are also fraught with a series of prion-specific problems. The biggest trouble, again, derives directly from the peculiar biology of TSE agents. As explained above, PrP^{Sc} possesses the same chemical composition as PrP^{C}, and the latter is a membrane-bound protein that is normally found in many cell types of healthy individuals including white blood cells (Aguzzi and Weissmann, 1997). Although PrP^{C} and PrP^{Sc} differ in a number of physical properties, it appears to be extremely difficult to develop immunological reagents which reliably differentiate between these two isoforms. Only one monoclonal antibody has been described to react with PrP^{Sc} but not with PrP^{C}, and its practical usefulness remains to be demonstrated since fourteen months after its publication no follow-up studies have appeared and even the company which developed this reagent in the first place does not appear to use it in its in-house screening assay for BSE prions.

The hitherto best method for the detection of prions is by performing Western blot analysis with homogenized brain tissue that has been digested with proteinase K (PK). The digestion is necessary since for Western blot analysis the secondary structure is broken up so that no difference is found any more between cellular prions (PrP^{C}) and pathological prions (PrP^{Sc}), however, while PrP^{C} is readily digested by PK under specified conditions, PrP^{Sc} is only degraded to relatively large fragments called PrP^{27-30}.

It is also already known to concentrate proteins by adsorbing them to so called magnetic beads (MB) to which a specific antibody is bound. However, the application of such a concentration method to PrP has been assumed to be impossible due to the specific features of prions.

Thus, still a great need exists to have a sensitive method or test to detect small amounts of prions not only for diagnosis but also for further investigating the disease, as well as agents to perform such tests.

Brief Description of the Invention

Hence it is one object of the present invention to provide a method for the detection of the pathological prion protein as PrP^{Sc} or PrP^{27-30}, respectively.

It is another object of the present invention to provide a method for searching for prion interacting agents.

Still another object of the present invention are agents recognizing PrP^{Sc} and/or PrP^{27-30}.

Still another object of the present invention are solid phase materials such as e.g. magnetic beads carrying such agents and composition comprising same.

Still another object of the present invention are compositions comprising such agents for purifying body fluids and sterilization of surgical and diagnostic instruments.

In the method of the present invention for the concentration of PrP^{Sc} or digestion products thereof, a body fluid, such as e.g. blood, urine, cerebrospinal fluid etc., or fluidized organ, such as brain tissue, lymph nodes, tonsils etc., is treated with a solid phase material such as magnetic beads(MB) whereby at least part of said material or beads, respectively, carries a prion

binding site. A preferred prion binding site is a factor with prion binding activity (PrPB).

The method works very well with a fluidized organ, in particular homogenized tissue of central nervous system, preferably homogenized brain tissue.

In several cases the prion binding site can only distinguish PrP^C and PrP^{Sc} in digested form. For such cases, it is necessary to digest the fluid or fluidized organ prior to the actual concentration step. A suitable digestion is obtained by digestion with proteinase K (PK), whereby it is important to inactivate the proteinase K prior to the addition of the solid phase, e.g. MBs.

Preferred solid phase materials carrying PrPB are prepared by coupling such materials with blood serum, or blood plasma, such as fresh frozen plasma of mammals, whereby an excess of protein is present during the coupling procedure. Such factors are designated spPrPB (s= serum and p= plasma) (see below). Even more preferred solid materials carrying PrPB are prepared by coupling purified plasminogen or fibrinogen to the beads(see below).

Very suitable solid materials are magnetic beads since they can easily be treated with specific components of interests and easily be collected by applying a magnetic field.

A further method of the present invention concerns the detection (and optionally quantification) of PrP^{Sc} or digestion products thereof, wherein PrP^{Sc} is first concentrated as described above, optionally also with previous digestion of the fluid or fluidized organ, and then detected and optionally compared with a standard. A suitable detection method is Western blot analysis. Such test may furthermore be embodied by other detection methods such as a microtiter plate format immunoassay (e.g. ELISA assay), an immunoprecipitation

assay, a BIACORE assay, immunocytochemical assay, histoblot assay etc.

Besides of the above mentioned methods, the present invention also concerns factors with prion binding activities such as sPrPBII, which is a prion binding activity in fraction II of ammonium sulfate precipitation of serum or pPrPBII which is a factor with prion binding activity in fraction II of ammonium sulfate precipitation of normal or fresh frozen plasma. Said factors are of course subject matter of the present invention in any form, such as in isolated form, or as ingredient in a composition, e.g. in a fraction of ammonium sulfate precipitation.

Said factors can be obtained by concentration and/or isolation of PrPBs whereby serum or plasma is subjected to fractionated ammonium sulfate precipitation thus that a PrPB of interest is precipitated, preferably in only one fraction. A further purification can be obtained by the application of further protein isolation methods.

The factors of the present invention are not only suitable for the detection of prions, but they have further applications in methods for the purification and removal of pathological prion protein from body fluids and organs, such as blood, urine, cerebrospinal fluid, brain tissue, lymph nodes, tonsils etc., or for the sterilization of surgical and/or diagnostic tools, basing on the affinity of PrPB for the pathological prion protein. They are furthermore tools for a therapy regimen based on the modulation of production of PrPB for preventing the spread of prions in the body. Especially suitable in this respect is plasminogen, that is also especially suitable for the purification of body fluids, e.g. blood units. Such purification may e.g. be performed by treating fluids with PrPBIp, in particular with immobilized plasminogen or plasma fractions containing same.

Also part of the present invention is a test for the detection of pathological prion protein in body fluids or organs such as blood, urine, cerebrospinal fluid, brain tissue, lymph nodes, tonsils etc, that utilizes the specific binding properties of PrPB to pathological prion protein. Such test can be embodied as a microtiter plate format immunoassay, e.g. ELISA assay, an immunoprecipitation assay, a BIACORE assay, immunogytochemical assay, histoblot assay etc.

Also the DNA sequences specific for biosynthesis of PrPB are comprised by the present invention as well as vectors able to express such DNA sequences in suitable hosts.

Furthermore comprised by the present invention are: a method for purification of PrPB by using PrP^{27-30} as bait; monoclonal and polyclonal antibodies produced in animals such as mice, rabbits, chicken etc., and directed against PrPB; single-chain Fv fragments and other types of fragments of antibodies produced in recombinant phages or in other recombinant systems, and directed against PrPB; a test predictive of susceptibility to prion diseases based on polymorphisms of PrPB, or on variations in the strength and pattern of production of PrPB; a transgenic animal, e.g. mouse that overproduction PrPB in brain, lymph nodes, or other organs, to be used in a bioassay for prions; a knockout animal, in particular a mouse, which is devoid of PrPB, to be used in a bioassay for prions; a production method of PrPB by expressing a DNA sequence specific for the biosynthesis of PrPB in a suitable host cell, such as bacteria, yeast, fungi, or eukaryotic cells, and by purification of PrPB from the aforementioned organisms; a use of natural or synthetic, preferably purified PrPB as a medicament for therapeutical applications in humans and animals; a vaccination of organisms with natural or synthetic PrPB, in particular plasminogen; a diagnostic

assay for human and/or animal diseases resulting from abnormal production and/or metabolism of PrPB.

Brief Description of the Drawings

Figure 1 is a scheme showing the IAP method.

Figure 2 shows Western Blots and IAP experiments of dilution experiments, whereby lanes 1 to 6 and 10 represent usual Western Blots and lanes 7 to 9 and 11 to 13 represent immuno affinity purification (IAP).

Figure 3 is a scheme showing the prion affinity assay (PAA) method.

Figure 4 represents Western Blots showing positive and begative controls of the PAA.

Figure 5 shows the observation that beads coated with sheep anti mouse IgG Abs by DYNAL bind PrPSc but not PrP27-30. Upon preincubation with normal mouse serum PrP27-30 is also bound.

Figure 6 represents Western Blots showing the results with serum proteins that are coupled to beads. The * means that the coupling was performed in the presence of an excess of proteins.

Figure 7 shows the effect of the addition of PK-treated brain homogenate to the assay.

Figure 8 represents Western Blots showing the results with PrP-deficient material.

Figure 9 represents Western Blots showing PAA of ammonium sulfate precipitates.

Figure 10 represents Western Blots showing PAA of ammonium sulfate precipitates that are not covalently crosslinked to the beads.

Figure 11 shows the result of the PAA of the 58 fractions of human plasma that were obtained by chromatography and differential precipitation and tested for binding activity.

Figure 12 represents Western Blots showing the results with purified plasminogen and fibrinogen.

Figure 13 represents Western Blots showing the calcium dependency of the binding activity of plasminogen and fibrinogen.

Figure 14 represents Western Blots showing the dependency of the binding activity of plasminogen on the native state of the proteins.

Figure 15 represents Western Blots showing PAA of plasminogen that is not covalently crosslinked to the beads.

Figure 16 shows the concepts of the bioassay.

Figure 17 shows the results of the bioassay.

Detailed Description of the Invention

As already mentioned above, there is a great need for a detection method for low concentrations of PrP^{Sc} that can be used as a diagnostic test for transmissible spongiform encephalopathies (TSEs).

There are basically three diagnostic principles for TSEs: histopathological detection of the typical spongiform changes in the CNS, detection of the scrapie-specific isoform of the prion protein, and the bioassay that detects infectivity. All these methods have limitations: histopathology is not useful for preclinical diagnosis since the structural changes appear late in the incubation period. Detection of the scrapie the Western specific is form of prion protein is more sensitive but still much less sensitive than the bioassay. The bioassay can, in principle, detect as little as 1 infectious unit but can last months or even years.

The hitherto used Western blot technique is based on the partial protease resistance of PrP^{Sc} that allows to distinguish between PrP^{C} and PrP^{Sc}. After protease treatment, PrP^{27-30} - the protease resistant core of PrP^{Sc} - can be detected but not PrP^{C} which is completely digested.

Although due to the "stickiness" of prions it was generally assumed that immuno affinity purification (IAP) cannot be applied, it has now been found that concentration can be achieved by applying magnetic beads (MB) carrying a prion binding site, preferably a factor with prion protein binding activity (PrPB).

Thus, because the sensitivity of detection of absolute amounts of PrP^{27-30} is a function of antibody affinity, and cannot be easily increased for each given antibody, in the scope of the present invention, despite of the hitherto assumed problems, first an "immuno affinity purification" (IAP) assay has been developed, using antibodies covalently crosslinked to solid phase material, e.g. magnetic beads. Because the monoclonal antibody (6H4 purchased at Prionics, Zurich, Switzerland, described in Korth et al., 1997), originally used for the development of the IAP, is not able to distinguish between PrP^{C} and PrP^{Sc} (it binds both undigested forms as well as digested PrP^{Sc}, i.e. PrP^{27-30}), it is necessary to perform Proteinase K digestion prior to the IAP (see Fig.1).

For the development of the present IAP method, the following model system was used: Two tests were performed to determine the efficiency of the method. On the one hand, small amounts of a scrapie-infected mouse brain homogenate were diluted with water and then subjected to the PrP^{Sc} concentration method. On the other hand, small amounts of a scrapie-infected brain homogenate was diluted with brain homogenate of non-infected mice in order to simulate a real situation in which a brain homogenate contains low amounts of PrP^{Sc} (see Fig.2).

In Figure 2, lanes 1 to 6 and 10 represent usual Western Blots and lanes 7 to 9 and 11 to 13 represent immuno affinity purification (IAP). PrnP% is material from PrP deficient mice. MB are of course only used for IAP whereby 6H4 refers to MB coupled with 6H4

antibodies and - refers to uncoupled MBs. PRP^C refers to brain homogenate of non-infected mice and PrP^{Sc} refers to brain homogenate of scrapie-infected mice. PK refers to Proteinase K digestion whereby - refers to non digestion and + to digested homogenate. The same abbreviations are used for the following figures.

For prion analysis in homogenate, in particular of brain tissue, it is important to use in a first homogenation step low concentration of ionic detergent, followed by low speed centrifugation, preferably 500 g 30 minutes, 4°C applied twice. For following steps high concentration of non-ionic detergent is used and a protein concentration of the homogenate of at most 5 mg/ml.

Conditions for the proteinase K digestion are preferably 50 µg/ml PK, 37°C and at least half an hour.

Suitable incubation conditions for the beads with homogenate are e.g. about 1.5 hours at room temperature, whereby for low concentrations longer incubation times might be preferable.

The concentration step in said first attempt was carried out by adding to digested homogenate magnetic beads (MB) carrying said 6H4.

If a digestion step is needed, it has to be performed prior to the concentration step, whereby the digestion, usually by proteinase K, has to be stopped prior to the concentration step by deactivating the proteinase e.g. with phenyl methyl sulfonyl fluoride or another agent known to the skilled person.

By applying the method of the present invention for e.g. brain tissue homogenate, PrP^{27-30} can be concentrated up to amounts detectable by Western blot analysis from tissue comprising much less pathological prion protein than needed for the hitherto known tests.

Using largely the same procedure, the above described method can also be applied as prion affinity assay (PAA) by exchanging the monoclonal antibody 6H4 by

other substances to be examined, for example in order to find a binding partner for PrP^{Sc} (see Fig.3).

As a positive control of this assay 6H4 (see Figure 4, lanes 1-3) is used and as a negative control mouse IgG or mouse albumin (see Figure 4, lanes 4-9).

In order to investigate whether a given mouse serum containes IgG that specifically recognize PrP^{Sc} magnetic beads that are already coated by the company DYNAL with sheep antibodies directed against mouse IgGs were used after preincubation with mouse serum. These beads - used without preincubation - were the first negative control (see Figure 5, lanes 1-2). As a second negative control these beads preincubated with normal mouse serum were used in order to show that IgGs from normal mouse serum do not bind to any form of PrP (see Figure 5, lanes 3-4). Surprisingly the beads alone showed an affinity to PrP^{Sc} but not to PrP^{27-30}. Upon preincubation with normal mouse serum also PrP^{27-30} is bound. Therefore it was hypothised that the sheep antibodies from DYNAL recognize a molecule that is associated with PrP^{Sc} but digested away after PK-treatment. As PrP^{27-30} is bound upon preincubation with normal mouse serum, this serum might contain the molecule with affinity to PrP^{Sc}.

The beads coupled to total mouse serum proteins did not show any affinity to any form of PrP. However, if the coupling of the total serum was performed in the presence of an excess of protein the beads showed the same binding to PrP^{27-30} as the monoclonal antibody 6H4 (see Figure 6, lanes 4-6) whereas the beads that were coupled in the presence of an excess of albumine still did not show any affinity to any form of PrP (see Figure 6, lanes 1-3). Though it was not possible to measure any difference of the coupling efficency of the two conditions it might be that offering an excess of proteins causes a sponge on the surface of the beads that

binds PrP^{27-30}. We also checkled whether PK-treated brain homogenate might enhance the binding as in the case of bound PrP^{27-30} total PK-digested brain homogenate is present: the addition of PK-digested brain homogenate from wild-type C57BL/6 mice or *Prnp*$^{o/o}$ mice allowed to bind PrP^{Sc} in addition to PrP^{27-30} (see Figure 7, lanes 1-3); the addition of inactive PK had no influence on the binding activity (see Figure 7, lanes 7-9). If coupled in the presence of an excess the activity of binding PrP^{27-30} was also found in the serum of man, sheep, cow and in the serum of terminally scrapie-sick C57BL/6 mice (data not shown).

Apart from an artefact it might well be that serum of several species contains activities (collectively termed PrP^{B}) that interact specifically with the pathogenic isoform of the prion protein and that are kinetically favoured in binding to the beads. The affinity to PrP^{27-30} could then be understood assuming that native PrP^{Sc} present in sick mice is saturated with PrP^{B} which might be released upon proteolytic digest. Alternatively, partial proteolysis may expose PrP^{B} binding sites on PrP^{Sc}. However, the fact that the addition of PK-treated brain homogenate allows to bind PrP^{Sc} indicates that there might be several different interactions leading to our observations.

The template-directed refolding hypothesis predicts that PrP^{C} and PrP^{Sc} form heterodimers during the conversion process. Therefore we investigated whether PrP^{B} is identical with PrP^{C}. However, when coupling in excess PrP^{B} activity was present in the serum of *Prnp*$^{o/o}$ mice at levels similar to those of wild-type mice, implying that PrP^{C} does not contribute to the binding activity (see Figure 8).

If PrP^{B} activity is not only caused by the special coupling conditions, it should be possible to "purify" it by fractionating mouse serum by differential ammonium sulfate precipitation. Indeed, it was possible

to precipitate PrP^B at an ammonium sulfate saturation below 50% whereby coupling of each fraction was performed in the presence of an excess of protein (see Figure 9). While purified rabbit immunoglobulins against total mouse serum did not contain PrP^B (data not shown), they efficiently bound PrP^{27-30} upon preincubation with full mouse serum (see Figure 10, lanes 1-3) or with proteins precipitating between 25% and 50% ammonium sulfate saturation (see Figure 10, lanes 4-6). Preincubation with proteins precipitating between 75% and 100% ammonium sulfate saturation did not lead to PrP^B activity (see Figure 10, lanes 7-9). This finding is important as it shows that the PrP^B activity is a property of one or more serum proteins independent of the covalent crosslink to the surface of the beads.

As the ammonium sulfate fractionation worked with human serum as well (data not shown), 58 fractions of human plasma were obtained by chromatography and differential precipitation and tested for binding activity to form an idea of the identity of PrP^B. All fractions were not coupled in the presence of an excess of proteins. Therefore the results can directly be compared with 6H4 or mouse IgG. 20 fractions tested positive: Plasminogen, fibrinogen, antithrombin III, antithrombin III heparin complex, C1 esterase inhibitor, factor IX and several fractions containing protein mixtures (see Figure 11). Purified plasminogen and also purified fibrinogen bound PrP^{Sc} in addition to PrP^{27-30} (see Figure 12). Out of the 38 fractions that tested negative, 6 contained purified proteins: Prothrombin complex concentrate, albumin, activated prothrombin complex concentrate, factor XIII and thrombin.

As mentioned, there are some hints that the binding of PrP^{27-30} is caused by different effects. The activity that binds PrP^{27-30} is termed $spPrP^B$ (s=serum and p=plasma) as it is present in serum and in plasma. Said activity is comparable to the activity found for

plasminogen and fibrinogen. Plasminogen and fibrinogen were furthermore characterized as they both bind also PrP^{Sc}.

As calcium is an important cofactor in the coagulation cascade it was investigated whether PrP^{B} activity is still intact if coagulation is inhibited by complexing calcium. In the presence of 10 mM EDTA the pathogenic PrP^{Sc} and PrP^{27-30} were still bound by plasminogen (see Figure 13, lanes 1-3) but only PrP^{27-30} by fibrinogen (see Figure 13, lanes 4-6). At least in the case of plasminogen this finding speaks against the possibility that the PrP^{B} activity is due to unspecific coagulation. Because PrP^{B} selectively interacts with the pathogenic PrP but not with PrP^{C}, interaction may be conformation-specific. When the assay was carried out in the presence of 6M urea the fraction containing purified plasminogen didn't bind PrP^{Sc} nor PrP^{27-30} (see Figure 14, lanes 8-9) under these conditions PrP^{Sc} becomes protease-sensitive (see Figure 14, lanes 14-15). As the conformation of PrP^{Sc} is thought to be responsible for the PK resistancy we conclude from this experiment that the interaction of plasminogen and PrP^{Sc} is conformation-dependant.

Furthermore it could be shown that PrP^{B} activity of plasminogen is not dependent on the covalent crosslink to the beads by using magnetic beads coated with antibodies directed against plasminogen and preincubated with plasminogen (see Figure 15, lanes 3-4). There are two negative controls: 1. If beads coated with antibodies against plasminogen are not at all preincubated (see Figure 15, lanes 1-2) or preicubated with albumin (see Figure 15, lanes 5-6), the pathogenic isoform of PrP is not bound. 2. If beads coated with albumin are preincubated with plasminogen there is also no binding to the pathogenic isoform of PrP (see Figure 15, lanes 7-8).

Furthermore it could be shown that at least $spPrP^B$ does not only bind the pathogenic PrP but also infectivity. For this purpose we inoculated indicator tga20 mice i.c. with 0.2% of the paramagnetic beads before eluting the other 99% of the beads and performing a western blot (see Figure 16). The animals that were inoculated with beads that bind the pathogenic PrP did all develop the disease (see Figure 17, lanes 4,5 and 7).

Examples:

Example 1: IAP method

The IAP protocol is the following: Bring the brain tissue in a 15 ml FALCON tube, put it on ice and leave it there for all steps. Add Homogenate Buffer (0.5% DOC / 0.5% NP-40 in PBS) to get 10 % (w/v) homogenate. Pass the tissue through a 18 gauge needle and a 22 gauge needle by sucking up and down for 15 times each. Centrifuge the homogenate for 30 minutes at 500 g and 4°C. Keep the supernatant. Determine the protein concentration. Centrifuge the homogenate for 30 minutes at 500 g and 4°C. Keep the supernatant. If the protein concentration is higher than 10 mg/ml then bring the homogenate to a protein concentration of 10 mg/ml using the homogenate buffer. Bring the homogenate to a protein concentration of 5 mg/ml and 3% Tween 20 / 3% NP-40 all in PBS. Add to the tissue homogenate Proteinase K to get a final concentration of 50 µg/ml. Incubate for 60 minutes at 37°C. Add PMSF to get a final concentration of 5 mM. Add 0.25 volumes of IAP buffer (3% Tween 20 /3 % NP-40 in PBS). Resuspend the magnetic beads (covered with 6H4) according to the protocol described below) thoroughly. Pipette out 100 µl. Remove buffer. Add the homogenate to the beads and incubate the bead-sample mixture with continous mixing for 1.5 hours at room temperature. Collect the beads using the MPC (strong

magnet). Wash three times with 1 ml Washing Buffer (2% Tween 20 / 2% NP-40 in PBS) and once with 1 ml PBS by vortexing for 15 seconds at room temperature and by using the MPC. Spin down the beads, discard the remaining supernatant using again the MPC. Add 24 µl x Loading Buffer (50 mM Tris pH 6,8; 2% SDS; 0.01% bromphenol blue; 10% glycerol). Heat to 95°C for 5 minutes. If the samples are stored at -20°C then heat them again for 30 seconds at 95°C before performing SDS-PAGE followed by western Blot: Assemble the glass plates according to the manufacturer's instructions. Prepare in a Falcon tube the appropriate volume of the Resolving Gel (2.1 ml H_2O, 1.5 ml 40 % Acrylamid, 1.3 ml 1.5 M Tris pH 8.8, 50 µl 10 % SDS, 50 µl 10 % Ammoniumpersulfat, 2 µl TEMED). Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel (the length of the comb plus 1 cm). Using a pasteur pipette carefully overlay the acrylamide with water. Place the gel in a vertical position at room temperature. After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionized water to remove any unpolymerized acrylamide. Prepare in a Falcon tube the appropriate volume of the Stacking Gel (1.48 ml H2O, 0.25 ml 40 % Acrylamid, 0.25 ml 1.0 M Tris pH 6.8, 20 µl 10 % SDS, 20 µl 10 % Ammoniumpersulfat, 2 µl TEMED). Mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles. Place the gel in a vertical position at room temperature. After polymerization is complete (30 minutes), remove the Teflon comb carefully. Mount the gel in the electrophoresis apparatus. Add Running buffer to

the top and bottom reservoirs. Remove any (25 mM Tris, 250 mM glycine, 0.1 % SDS) bubbles that become trapped at the bottom of the gel between the glass plates. Load 24 µl of each of the samples in a predetermined order into the bottom of the wells (1. well: Low -range marker). Load an equal volume of 1x Gel-loading Buffer into any wells that are unused. Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom reservoir). Apply 10 V/cm to the gel. After the dye front has moved into the resolving gel (30 minutes), increase the voltage to 14 V(cm and run the gel until the bromophenol blue reaches the bottom of the resolving gel (1 hour). Then turn off the power supply. Cut six sheets of absorbent paper (Whatman 3MM or equivalent) and one sheet of nitrocellulose to the size of the gel (6cm x 8 cm). If the paper overlaps the edge of the gel, the current will short-circuit the transfer and bypass the gel, preventing efficient transfer. Wet the absorbent paper, the nitrocellulose and the gel by soaking in Transfer (39 mM glycine, 48 mM Tris, 0.037 % SDS, 20 % methanol) Buffer. On the bottom plate of the apparatus (the anode), assemble the gel, nitrocellulose, and paper in this order:

bottom electrode,

three layers absorbent paper soaked in transfer buffer,

one nitrocellulose membrane soaked in transfer buffer,

polyacrylamide gel slightly wetted with transfer buffer,

three layers absorbent paper soaked in transfer buffer.

Check carefully for air bubbles and gently remove them either by using a gloved hand or by rolling a pipet over the sandwich. Dry any buffer that may surround the gel-paper sandwich. Carefully place the upper

electrode (the cathode) on top of the stack. Put a weight on it. Connect the electrodes and commence transfer. Running time is 1 hour with a current of 1 mA/cm^2. After transfer, disconnect the power source. Carefully disassemble the apparatus. Mark membrane to follow orientation (usually by snipping off lower left-hand corner, the number one lane). Rinse the membrane three times with TBS-T. Add Blocking Buffer (5 % (w/v) nonfat dry milk in TBS-T). Incubate at room temperature with agitation for 30 minutes. Rinse the membrane three times with TBS-T. Add to 2.5 µl of mAB 6H4 (2 mg/ml) 12.5 ml of 1% (w/v) nonfat dry milk in TBS-T. Incubate at room temperature with agitation for 1 hour or overnight at 4°C. Remove the membrane from the antibody solution and wash three times for 10 minutes each in TBS-T. Add to 1.25 µl of relativ anti mouce IgG1-HRP 12.5 ml of 1% (w/v) nonfat dry milk in TBS-T. Incubate at room temperature with agitation for 1 hour. Remove the membrane from the antibody solution and wash three times for 15 minutes each in TBS-T. Mix 1 ml of detection solution 1 with 1 ml of detection solution 2 from the ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Incubate for precisely 1 minute at room temperature without agitation. Drain off excess detection reagent by putting the membrane on a absorbent paper. Gently place the membrane, protein side down, on a SaranWrap. Close SaranWrap to form a envelope avoiding pressure on the membrane. Place the membrane, protein side up, in the film cassette. Work as quickly as possible. Switch off the lights and carefully place a sheet of autoradiography film such as (Hyperfilm ECL) on top of the membrane, close the cassette and expose for some seconds (15", 30").

Example 2: PAA method

Couple the protein of interest to magnetic beads: Bring 100 µg of protein into approx. 1ml of Coupling Buffer (0.1 M borate buffer pH 9.5: dissolve 6.183 g H3BO3 in 800 ml distilled water, Adjust pH to 9.5 using 5 M NaOH and adjust volume to 1000 ml with distilled water; if necessary, change buffer by dialysis). (If coupling was performed in the presence of an excess, 1 mg was used for 1 ml of coupling buffer.) Make a homogeneous suspension of the Dynabeads M-280 Tosylactivated by Dynal using a pipette and by vortexing for approximately 1 min. Pipette out 1 ml of Dynabeads and wash as follows: Place the tube in the DYNAL MPC. Leave to separate for 2 minutes. Remove the supernatant taking care not to disturb the Dynabeads. Remove the tube from the Dynal MPC and resuspend the Dynabeads in PBS. Repeat these steps and resuspend the Dynabeads in the coupling buffer containing the antibodies. Incubate for 24 h at 37°C with tilt rotation. Place the tube in the magnet for 3 minutes and remove the supernatant. Wash the coated Dynabeads six times: 2 x in PBS/BSA (add 0.1 % (w/v) bovine serum albumin (final concentration) to PBS), pH 7.4 for 5 minutes at room temperature; 1 x in Blocking Buffer (0.2 M Tris pH 8.5 with 0.1 % (w/v) BSA: dissolve 2.42 g Tris in 80 ml distilled water. Adjust pH to 8.5 using 1 M HCl, add 0.1 % BSA and adjust volume to 100 ml with distilled water) for 4 h at 37°C; 1 x in PBS/BSA, pH 7.4 for 5 minutes at room temperature; 1 x in 1% Tween 20 for 10 minutes;1 x in PBS/BSA, pH 7.4 for 5 minutes at room temperature. Store the coated Dynabeads in PBS/BSA pH 7.4, 0.02% sodium azide. Then prepare Sample I: Add 1 ml of PAA Buffer (3 % NP-40 / 3 % Tween 20 in PBS) to 10 µl of not infected brain homogenate (Protein concentration 5 mg/ml; 0.5% DOC / 0.5 NP-40). Then prepare Sample II and III: Add 1 ml of PAA Buffer (3 % NP-40 / 3 % Tween 20 in PBS) to 10 µl of infected brain homogenate (Protein concentration 5 mg/ml; 0.5% DOC / 0.5 NP-40). Incubate Sample I and Sample II for 30 minutes at

37°C without PK. Incubate Sample III for 30 minutes at 37°C with PK at final concentration of 50 µg/ml (add 50 µl of PK 1mg/ml). Add PMSF to all samples to get a final concentration of 5 mM (add 50 µl of 100 mM PMSF). Resuspend the Magnetic Beads thoroughly. Pipette out 100 µl. Add the beads to the Samples and incubate the bead-sample mixture with continuous mixing for 1.5 hours at room temperature. Collect the beads using the MPC. Wash three times with 1 ml Washing Buffer and once with 1 ml PBS by vortexing for 15 seconds at room temperature and by using the MPC. Spin down the beads, discard the remaining supernatant using again the MPC. Add 24 µl 1 x Loading Buffer. Heat to 95°C for 5 minutes. If the samples are stored at -20°C then heat them again for 30 seconds at 95°C before loading on the gel.

As a positive control of this assay 6H4 is used and as a negative control mouse IgG or mouse albumin (see Figure 4).

Example 3

In order to investigate whether a given mouse serum containes IgG that specifically recognize PrP^{Sc} magnetic beads that are already coated by the company DYNAL with sheep antibodies directed against mouse IgGs were used after preincubation with mouse serum. These beads were the first negative control. As a second negative control these beads preincubated with normal mouse serum were used in order to show that IgGs from normal mouse serum do not bind to any form of PrP. Surprisingly the beads alone showed an affinity to PrP^{Sc} but not to PrP^{27-30}. Upon preincubation with normal mouse serum also PrP^{27-30} is bound (see Figure 5). Therefore it was hypothised that the sheep antibodies from DYNAL recognize a molecule that is associated with PrP^{Sc} but digested away after PK-treatment. As PrP^{27-30} is bound upon preincubation with normal mouse serum, this serum might contain the molecule with affinity to PrP^{Sc}.

Example 4

The beads coupled to total mouse serum proteins did not show any affinity to any form of PrP. However, if the coupling of the total serum was performed in the presence of an excess of protein the beads showed the same binding to PrP^{27-30} as the monoclonal antibody 6H4 whereas the beads that were coupled in the presence of an excess of albumine still did not show any affinity to any form of PrP (see Figure 6). Though it was not possible to measure any difference of the coupling efficency of the two conditions it might be that offering an excess of proteins causes a sponge on the surface of the beads that binds PrP^{27-30}.

Example 5

We also checkled whether PK-treated brain homogenate might enhance the binding as in the case of bound PrP^{27-30} total PK-digested brain homogenate is present: the addition of PK-digested brain homogenate from wild-type C57BL/6 mice or *Prnp*$^{o/o}$ mice allowed to bind PrP^{Sc} in addition to PrP^{27-30}; the addition of inactive PK had no influence on the binding activity (see Figure 7).

Example 6

If coupled in the presence of an excess the activity of binding PrP^{27-30} was also found in the serum of man, sheep, cow and in the serum of terminally scrapie-sick C57BL/6 mice (data not shown).

Example 7

The template-directed refolding hypothesis predicts that PrP^{C} and PrP^{Sc} form heterodimers during the conversion process. Therefore we investigated whether PrP^{B} is identical with PrP^{C}. However, when coupling in excess PrP^{B} activity was present in the serum of *Prnp*$^{o/o}$

mice at levels similar to those of wild-type mice, implying that PrP^C does not contribute to the binding activity (se Figure 8).

Example 8

If PrP^B activity is not only caused by the special coupling conditions, it should be possible to "purify" it by fractionating mouse serum by differential ammonium sulfate precipitation. Indeed, it was possible to precipitate PrP^B at an ammonium sulfate saturation below 50% whereby coupling of each fraction was performed in the presence of an excess of protein (see Figure 9). While purified rabbit immunoglobulins against total mouse serum did not contain PrP^B (data not shown), they efficiently bound PrP^{27-30} upon preincubation with full mouse serum or with proteins precipitating between 25% and 50% ammonium sulfate saturation. Preincubation with proteins precipitating between 75% and 100% ammonium sulfate saturation did not lead to PrP^B activity (see Figure 10). This finding is important as it shows that the PrP^B activity is a property of one or more serum proteins independent of the covalent crosslink to the surface of the beads.

Example 9

As the ammonium sulfate fractionation worked with human serum as well (data not shown), 58 fractions of human plasma were obtained by chromatography and differential precipitation and tested for binding activity to form an idea of the identity of PrP^B. All fractions were not coupled in the presence of an excess of proteins. Therefore the results can directly be compared with 6H4 or mouse IgG. 20 fractions tested positive: Plasminogen, fibrinogen, antithrombin III, antithrombin III heparin complex, C1 esterase inhibitor, factor IX and several fractions containing protein mixtures (see Figure 11). Purified plasminogen and also

purified fibrinogen bound PrP^{Sc} in addition to PrP^{27-30} (see Figure 12). Out of the 38 fractions that tested negative, 6 contained purified proteins: Prothrombin complex concentrate, albumin, activated prothrombin complex concentrate, factor XIII and thrombin.

Example 10

As calcium is an important cofactor in the coagulation cascade it was investigated whether PrP^{B} activity is still intact if coagulation is inhibited by complexing calcium. In the presence of 10 mM EDTA the pathogenic PrP^{Sc} and PrP^{27-30} were still bound by plasminogen but only PrP^{27-30} by fibrinogen (Fig. 13). At least in the case of plasminogen this finding speaks against the possibility that the PrP^{B} activity is due to unspecific coagulation.

Example 11

Because PrP^{B} selectively interacts with the pathogenic PrP but not with PrP^{C}, interaction may be conformation-specific. When the assay was carried out in the presence of 6M urea the fraction containing purified plasminogen didn't bind PrP^{Sc} nor PrP^{27-30}; under these conditions PrP^{Sc} becomes protease-sensitive (Fig. 14). As the conformation of PrP^{Sc} is thought to be responsible for the PK resistancy we conclude from this experiment that the interaction of plasminogen and PrP^{Sc} is conformation-dependant.

Example 12

Furthermore it could be shown that PrP^{B} activity of plasminogen is not dependent on the covalent crosslink to the beads by using magnetic beads coated with antibodies directed against plasminogen and preincubated with plasminogen (Fig. 15).

Example 13

Furthermore it could be shown that at least $spPrP^B$ does not only bind the pathogenic PrP but also infectivity. For this purpose we inoculated indicator tga20 mice i.c. with 0.2% of the paramagnetic beads before eluting and performing a western blot. The animals that were inoculated with beads that bind the pathogenic PrP did all develop the disease (Fig. 16, Fig. 17).

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

References

Aguzzi, A. (1997). Neuro-immune connection in spread of prions in the body? The Lancet 349, 742-743.

Aguzzi, A. (1998). Protein conformation dictates prion strain. Nat Med 4, 1125-6.

Aguzzi, A., Blättler, T., Klein, M. A., Räber, A. J., Hegyi, I., Frigg, R., Brandner, S., and Weissmann, C. (1997). Tracking prions: the neurografting approach. Cell Mol Life Sci 53, 485-95.

Aguzzi, A., and Collinge, J. (1997). Post-exposure prophylaxis after accidental prion inoculation. Lancet 350, 1519-20.

Aguzzi, A., and Weissmann, C. (1997). Prion research: the next frontiers. Nature 389, 795-798.

Aguzzi, A., and Weissmann, C. (1998). Spongiform encephalopathies. The prion's perplexing persistence. Nature 392, 763-4.

Aguzzi, A., and Weissmann, C. (1996). Spongiform encephalopathies: a suspicious signature. Nature 383, 666-7.

Bernoulli, C., Siegfried, J., Baumgartner, G., Regli, F., Rabinowicz, T., Gajdusek, D. C., and Gibbs, C. J. (1977). Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery [letter]. Lancet 1, 478-479.

Blättler, T., Brandner, S., Raeber, A. J., Klein, M. A., Voigtländer, T., Weissmann, C., and Aguzzi, A. (1997). PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. Nature 389, 69-73.

Brandner, S., Isenmann, S., Raeber, A., Fischer, M., Sailer, A., Kobayashi, Y., Marino, S., Weissmann, C., and Aguzzi, A. (1996). Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature 379, 339-43.

Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H., and Bostock, C. J. (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent [see comments]. Nature 389, 498-501.

Chazot, G., Broussolle, E., Lapras, C., Blattler, T., Aguzzi, A., and Kopp, N. (1996). New variant of Creutzfeldt-Jakob disease in a 26-year-old French man [letter]. Lancet 347, 1181.

Duffy, P., Wolf, J., Collins, G., DeVoe, A. G., Streeten, B., and Cowen, D. (1974). Possible person-to-person transmission of Creutzfeldt-Jakob disease. N Engl J Med 290, 692-3.

Fraser, H., Brown, K. L., Stewart, K., McConnell, I., McBride, P., and Williams, A. (1996). Replication of Scrapie in Spleens of Scid Mice Follows Reconstitution With Wild-Type Mouse Bone Marrow. Journal of General Virology 77, 1935-1940.

Gibbs, C. J., Jr., Joy, A., Heffner, R., Franko, M., Miyazaki, M., Asher, D. M., Parisi, J. E., Brown, P. W., and Gajdusek, D. C. (1985). Clinical and pathological features and laboratory confirmation of Creutzfeldt-Jakob disease in a recipient of pituitary-derived human growth hormone. N Engl J Med 313, 734-8.

Hill, A. F., Butterworth, R. J., Joiner, S., Jackson, G., Rossor, M. N., Thomas, D. J., Frosh, A., Tolley, N., Bell, J. E., Spencer, M., King, A., Al-Sarraj, S., Ironside, J. W., Lantos, P. L., and Collinge, J. (1999). Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. Lancet 353, 183-9.

Hill, A. F., Desbruslais, M., Joiner, S., Sidle, K. C., Gowland, I., Collinge, J., Doey, L. J., and Lantos, P. (1997). The same prion strain causes vCJD and BSE [letter] [see comments]. Nature 389, 448-50.

Hill, A. F., Zeidler, M., Ironside, J., and Collinge, J. (1997). Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. Lancet 349, 99.

Hilton, D. A., Fathers, E., Edwards, P., Ironside, J. W., and Zajicek, J. (1998). Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease [letter]. Lancet 352, 703-4.

Kitamoto, T., Muramoto, T., Mohri, S., Doh ura, K., and Tateishi, J. (1991). Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. J.Virol. 65, 6292-6295.

Klein, M. A., Frigg, R., Flechsig, E., Raeber, A. J., Kalinke, U., Bluethmann, H., Bootz, F., Suter, M., Zinkernagel, R. M., and Aguzzi, A. (1997). A crucial role for B cells in neuroinvasive scrapie. Nature 390, 687-90.

Klein, M. A., Frigg, R., Raeber, A. J., Flechsig, E., Hegyi, I., Zinkernagel, R. M., Weissmann, C., and Aguzzi, A. (1998). PrP expression in B lymphocytes is not required for prion neuroinvasion. Nat Med 4, 1429-33.

Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wuthrich, K., and Oesch, B. (1997). Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. Nature 390, 74-7.

Lasmezas, C. I., Cesbron, J. Y., Deslys, J. P., Demaimay, R., Adjou, K. T., Rioux, R., Lemaire, C., Locht, C., and Dormont, D. (1996). Immune system-dependent and -independent replication of the scrapie agent. J Virol 70, 1292-5.

O'Rourke, K. I., Huff, T. P., Leathers, C. W., Robinson, M. M., and Gorham, J. R. (1994). SCID mouse spleen does not support scrapie agent replication. J. Gen. Virol. 75, 1511 - 1514.

Raeber, A. J., Klein, M. A., Frigg, R., Flechsig, E., Aguzzi, A., and Weissmann, C. (1999). PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice. EMBO J 18, 2702-2706.

Schreuder, B. E., van Keulen, L. J., Smits, M. A., Langeveld, J. P., and Stegeman, J. A. (1997). Control of scrapie eventually possible? Vet Q 19, 105-13.

Schreuder, B. E., van Keulen, L. J., Vromans, M. E., Langeveld, J. P., and Smits, M. A. (1998). Tonsillar biopsy and PrPSc detection in the preclinical diagnosis of scrapie. Vet Rec 142, 564-8.

Vankeulen, L. J. M., Schreuder, B. E. C., Meloen, R. H., Mooijharkes, G., Vromans, M. E. W., and Langeveld, J. P. M. (1996). Immunohistochemical Detection of Prion Protein in Lymphoid Tissues of Sheep With Natural Scrapie. Journal of Clinical Microbiology 34, 1228-1231.

Weber, T., and Aguzzi, A. (1997). The spectrum of transmissible spongiform encephalopathies. Intervirology 40, 198-212.

Weissmann, C., and Aguzzi, A. (1997). Bovine spongiform encephalopathy and early onset variant Creutzfeldt- Jakob disease. Curr Opin Neurobiol 7, 695-700.

Wells, G. A., Hawkins, S. A., Green, R. B., Austin, A. R., Dexter, I., Spencer, Y. I., Chaplin, M. J., Stack, M. J., and Dawson, M. (1998). Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. Vet Rec 142, 103-6.

Will, R., Cousens, S., Farrington, C., Smith, P., Knight, R., and Ironside, J. (1999). Deaths from variant Creutzfeldt-Jakob disease. Lancet 353, 9157-9158.

Will, R., Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, and Smith (1996). A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347, 921-925.

Claims

1. A method for the concentration of PrP^{Sc} or digestion products thereof, wherein a body fluid or fluidized organ is treated with solid phase material such as e.g. magnetic beads (MB) whereby at least part of said material or beads, respectively, carries a prion binding site.

2. The method of claim 1, wherein the prion binding site is a factor with prion binding activity (PrPB).

3. The method of claim 1, wherein the fluidized organ is homogenized tissue of central nervous system.

4. The method of claim 1, wherein the fluidized organ is homogenized brain tissue.

5. The method of claim 1, wherein the fluid is a fluid that has been digested by proteinase K (PK).

6. The method of claim 2, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling MBs with blood serum, or blood plasma.

7. The method of claim 1, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling solid phase material with serum or plasma fraction II of ammonium sulfate precipitation.

8. The method of claim 1, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling MBs with material selected from the group consisting of plasma fraction I of ammonium sulfate precipitation, plasminogen and fibrinogen.

9. The method of claim 1, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling solid phase material with plasminogen, fibrinogen or sPrPBII or pPrPBII.

10. A method for the detection, and optionally quantification, of PrP^{Sc} or digestion products thereof, wherein PrP^{Sc} is first concentrated according to

one of claims 1 to 6 and then detected, and optionally compared with a standard.

11. The method of claim 10, wherein the detection is performed by Western blot analysis.

12. sPrPBII which is a factor with prion binding activity in fraction II of ammonium sulfate precipitation of serum.

13. pPrPBII which is a factor with prion binding activity in fraction II of ammonium sulfate precipitation of normal or fresh frozen plasma.

14. spPrPB which is a factor with prion binding activity in total serum or plasma.

15. Solid phase material, such as MBs, carrying a PrPB.

16. A composition for the purification of body fluids and/or the sterilization of surgical or diagnostic tools comprising a PrPB, preferably coupled to a solid phase material.

17. A method for the sterilization of surgical or diagnostic tools wherein said tools are treated with a PrPB comprising composition, preferably a composition comprising PrPB coupled to a solid phase material.

18. A method for the concentration and/or isolation of PrPBs wherein serum or plasma is subjected to fractionated ammonium sulfate precipitation thus that at least one PrPB of interest is precipitated, preferably in only one fraction.

19. The method of claim 18, wherein the PrPB comprising fraction is further purified by further protein isolation methods.

20. A method for purification and/or removal of pathological prion protein from body fluids or fluidized organs, such as blood, urine, cerebrospinal fluid, brain tissue, lymph nodes, tonsils, or for the sterilization of surgical and/or diagnostic tools basing

on the affinity of PrPB for the pathological prion protein.

21. A method for the purification of body fluids, e.g. blood units, wherein the fluid is treated with plasminogen.

22. A therapy regimen based on the modulation of production of PrPB for preventing the spread of prions in the body.

23. The regimen of claim 22, wherein the PrPB is plasminogen.

24. A test for the detection of pathological prion protein in body fluids or organs such as blood, urine, cerebrospinal fluid, brain tissue, lymph nodes, tonsils etc, that utilizes the specific binding properties of PrPB to pathological prion protein.

25. The test of claim 24 that is embodied as a microtiter plate format immunoassay, e.g. ELISA assay, an immunoprecipitation assay, a BIACORE assay, a immunocytochemical assay, a histoblot assay, etc.

26. A DNA sequence specific for biosynthesis of PrPB and/or an expression vector comprising same.

27. A method for purification of PrPB by using PrP^{27-30} as bait.

28. Monoclonal and polyclonal antibodies produced in animals, such as mice, rabbits, chicken, or other species, and directed against PrPB.

29. Single-chain Fv fragments and other types of fragments of antibodies produced in recombinant phages or in other recombinant systems, and directed against PrPB.

30. A test predictive of susceptibility to prion diseases based on polymorphisms of PrPB, or on variations in the strength and pattern of production of PrPB.

31. A transgenic animal, in particular mouse that overproduces PrPB in brain, lymph nodes, or other organs, to be used in a bioassay for prions.

32. A knockout animal, in particular a knockout mouse, which is devoid of PrPB, to be used in a bioassay for prions.

33. Production method of PrPB by expressing a DNA sequence specific for the biosynthesis of PrPB in a suitable host cell, such as bacteria, yeast, fungi, or eukaryotic cells, and by purification of PrPB from the aforementioned organisms.

34. Use of natural or synthetic PrPB as a medicament for therapeutical applications in humans and animals.

35. A vaccination of organisms with natural or synthetic PrPB, in particular PrPBIp.

36. A diagnostic assay for human and/or animal diseases resulting from abnormal production and/or metabolism of PrPB.

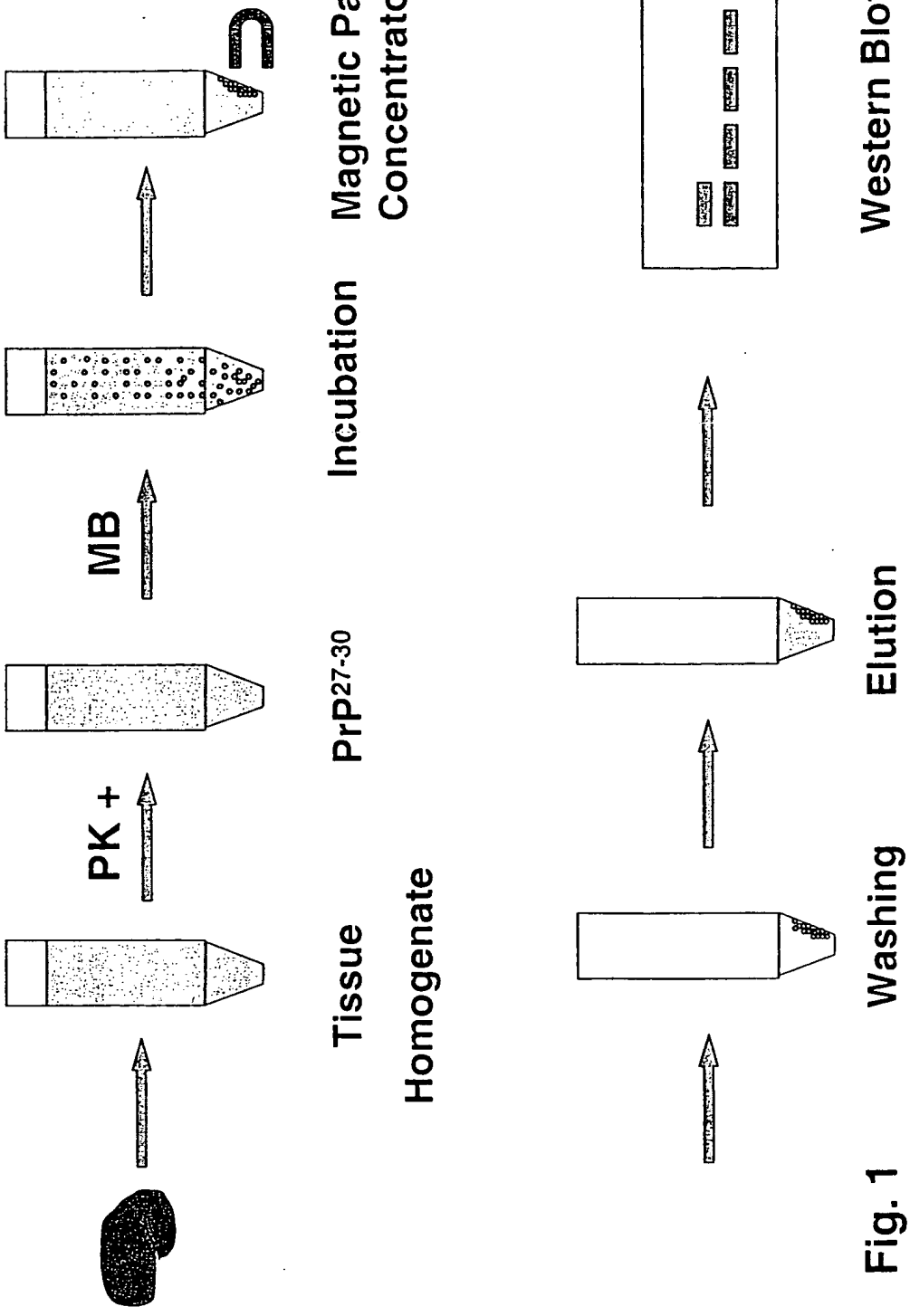

Tissue Homogenate
PK +
PrP27-30
MB
Incubation
Magnetic Particle Concentrator
Washing
Elution
Western Blot

Fig. 1

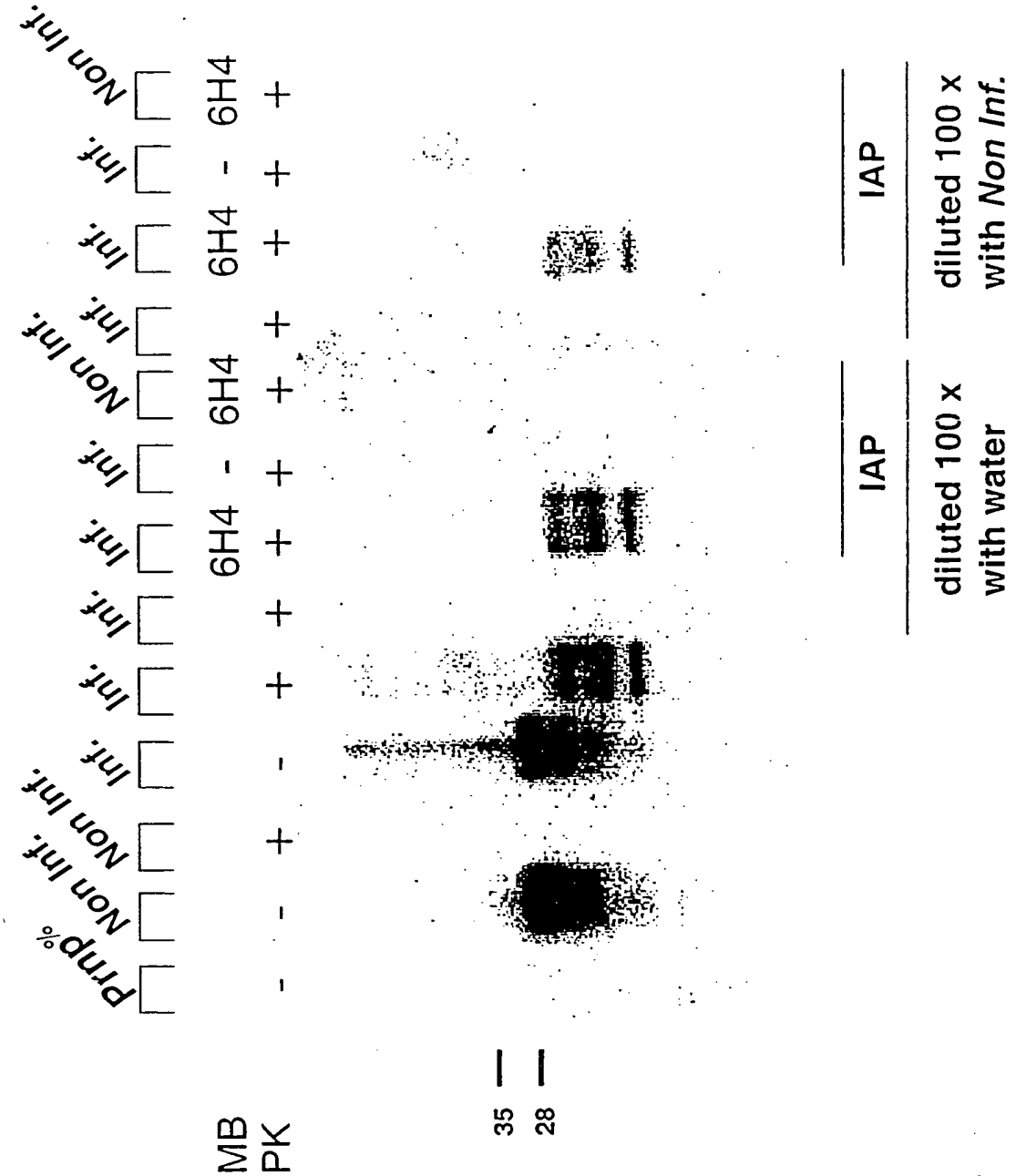

prnp%
Non Inf.
Non Inf.
Non Inf.
Inf.
Inf.
Inf.
Inf.
Inf.
Inf.
Non Inf.
Inf.
Inf.
Non Inf.
MB
PK
-
-
+
-
+
+
+
+
+
+
6H4
6H4
+
+
+
+
+
+
+
+
+
+
35
28
IAP
diluted 100 x
with water
IAP
diluted 100 x
with Non Inf.

Fig. 2

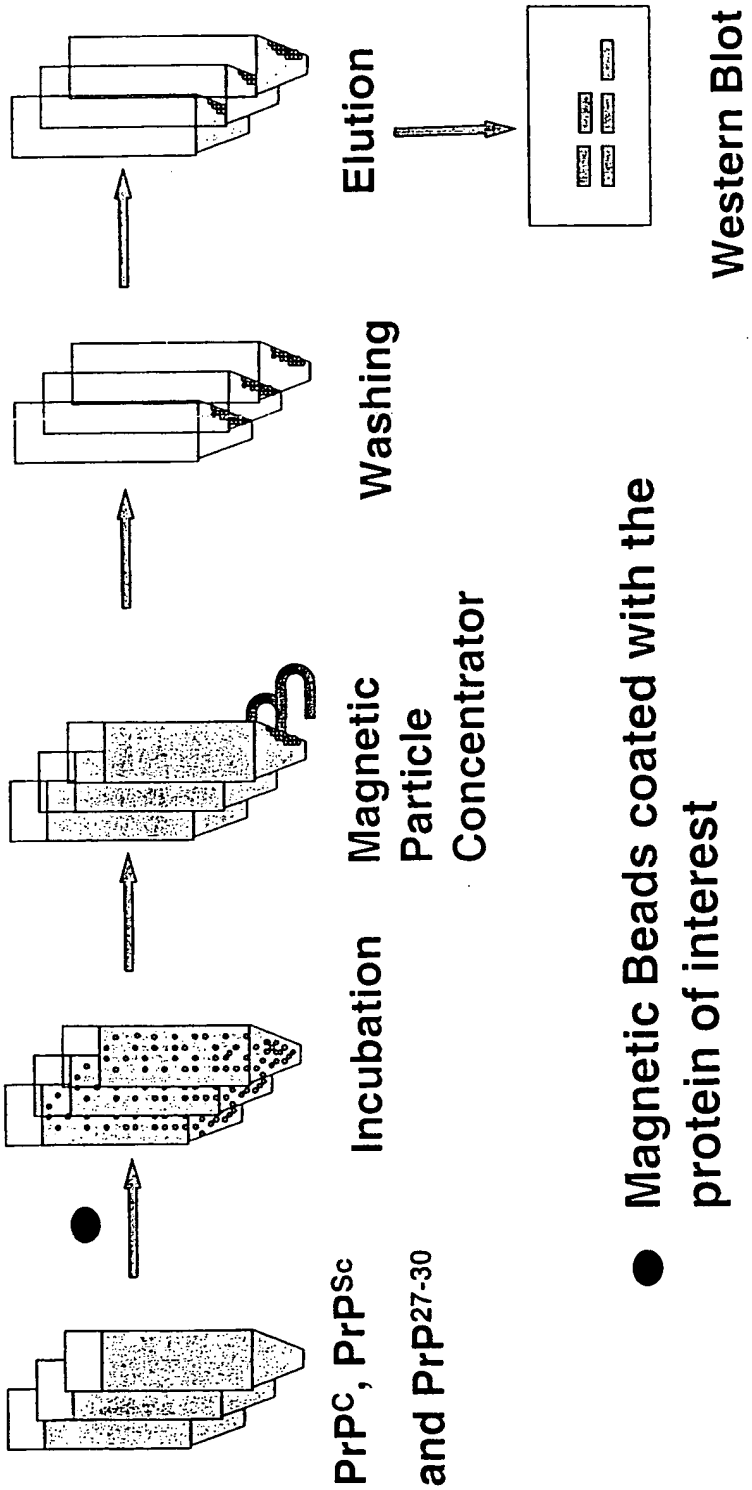

PrP^C, PrP^Sc and PrP^27-30
Incubation
Magnetic Particle Concentrator
Washing
Elution
Western Blot
Magnetic Beads coated with the protein of interest

Fig. 3

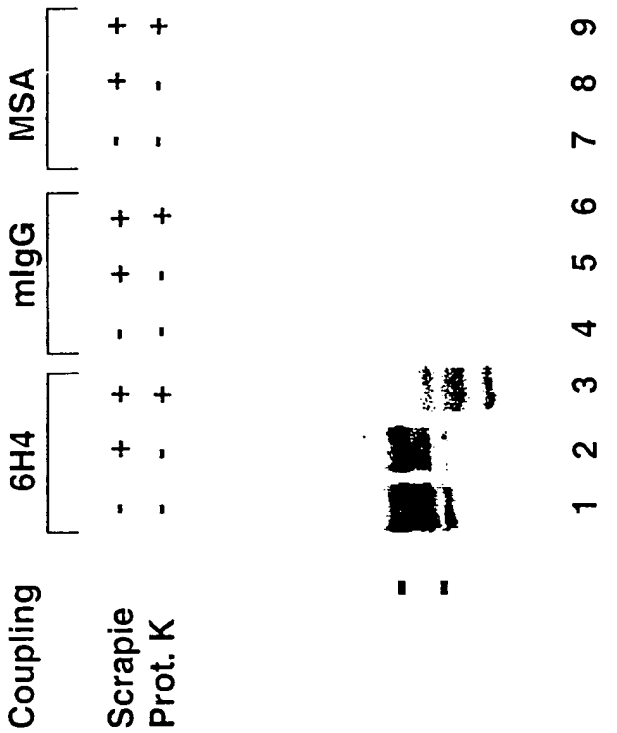


Fig. 4

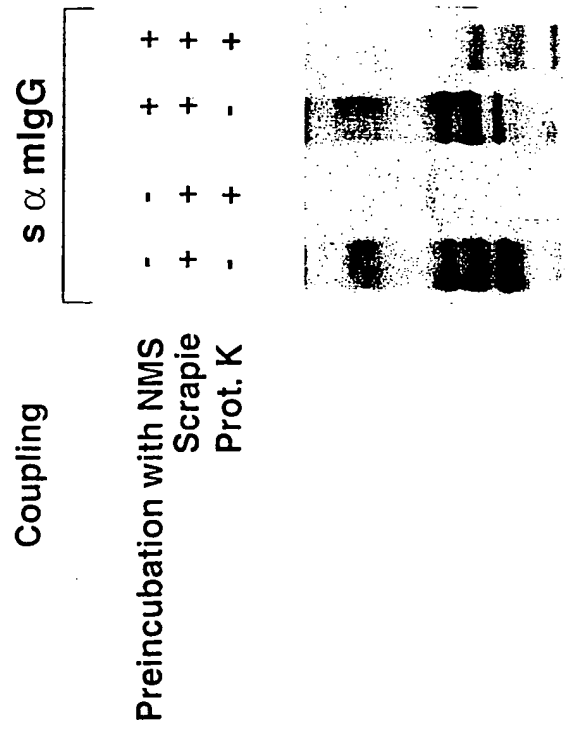


Fig. 5

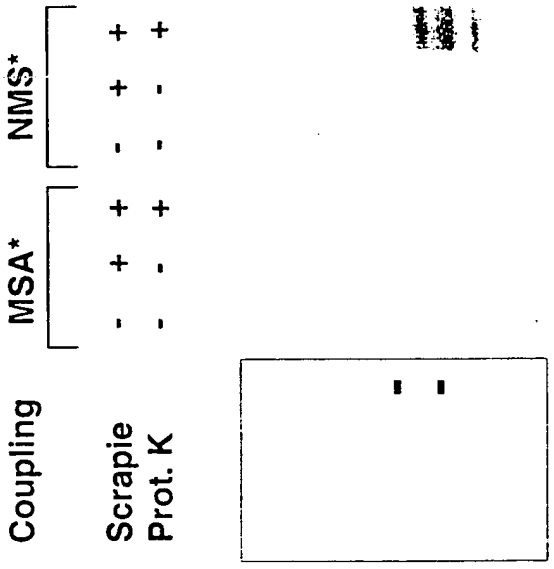


Fig. 6

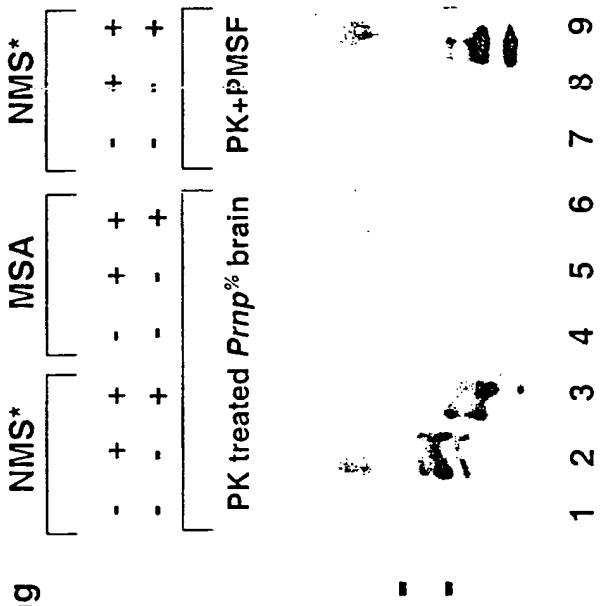

Coupling
NMS*
MSA
NMS*
Scrapie
Prot. K
- + + - + +
- - + - - +
PK treated Prnp% brain
PK+PMSF
1 2 3 4 5 6 7 8 9

Fig. 7

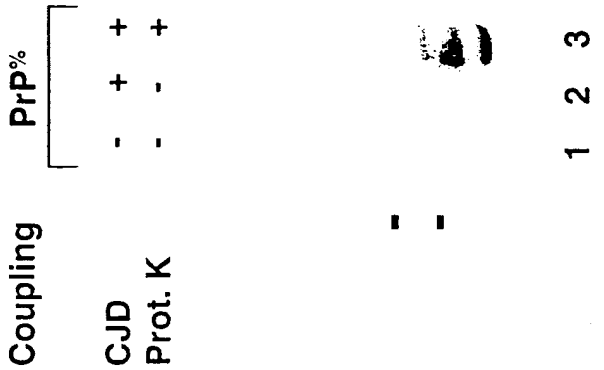


Fig. 8

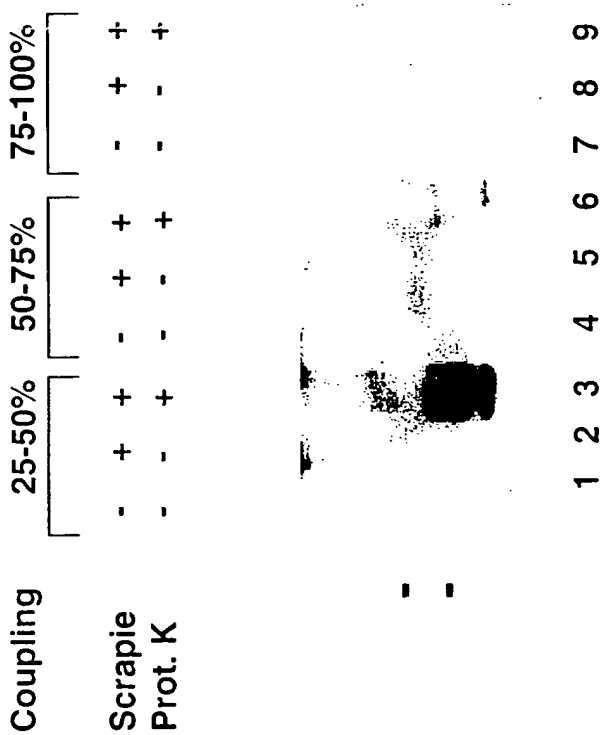


Fig. 9

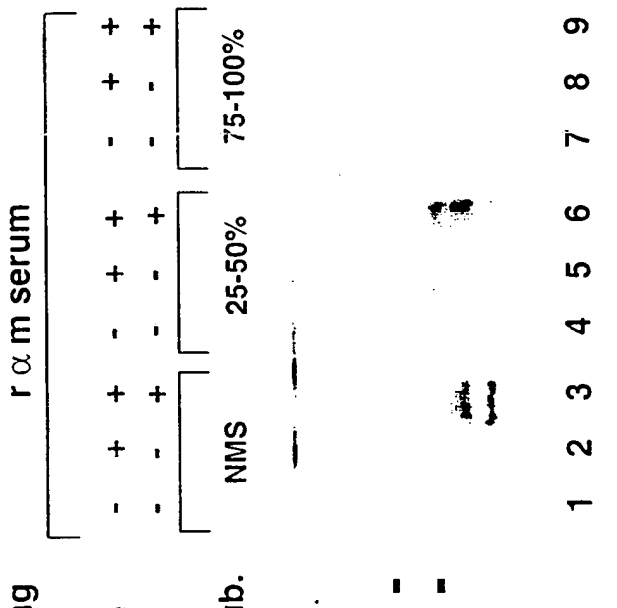

Coupling
r α m serum
Scrapie
Prot. K
Preincub.
NMS
25-50%
75-100%
1 2 3 4 5 6 7 8 9

Fig. 10

Fraction	PrP^{C}	PrP^{Sc}	PrP^{27-30}
Plasminogen	-	+	+
Fibrinogen	-	+	+
Antithrombin III	-	-	+
C1 esterase inhibitor	-	-	+
Antithrombin III heparin complex	-	-	+
Plasma pool	-	-	(+)
Factor IX, Fibrin sealant 2 (!)	-	-	+
12 fractions containing protein mixtures	-	-	+
Total: 20 positive fractions			
Prothrombin complex concentrate	-	-	-
Human albumin	-	-	-
Activated prothrombin complex concentrate	-	-	-
Factor VIII concentrate	-	-	-
Human Thrombin	-	-	-
Factor XIII	-	-	-
Factor IX concentrate, Fibrin sealant 1 (!)	-	-	-
30 fractions containing protein mixtures	-	-	-
Total: 38 negative fractions			

Fig. 11

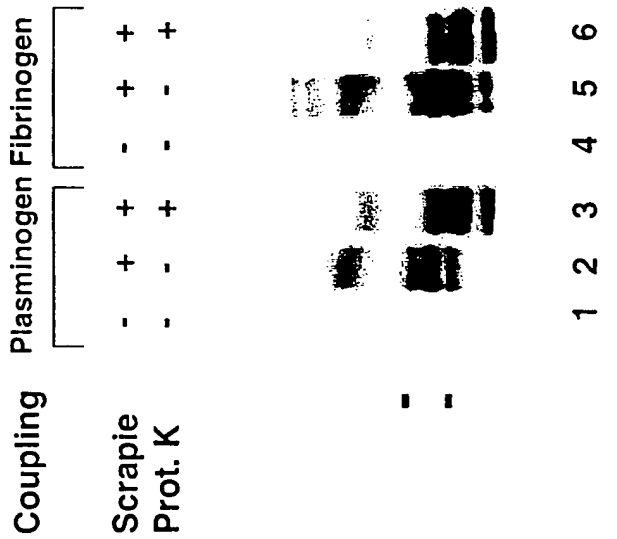


Fig. 12

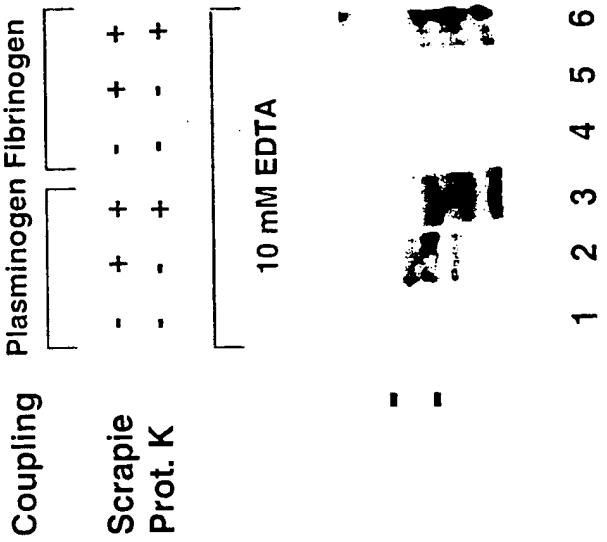

Coupling
Plasminogen Fibrinogen
Scrapie
Prot. K
10 mM EDTA
- + + - + +
- - + - - +
- -
1 2 3 4 5 6

Fig. 13

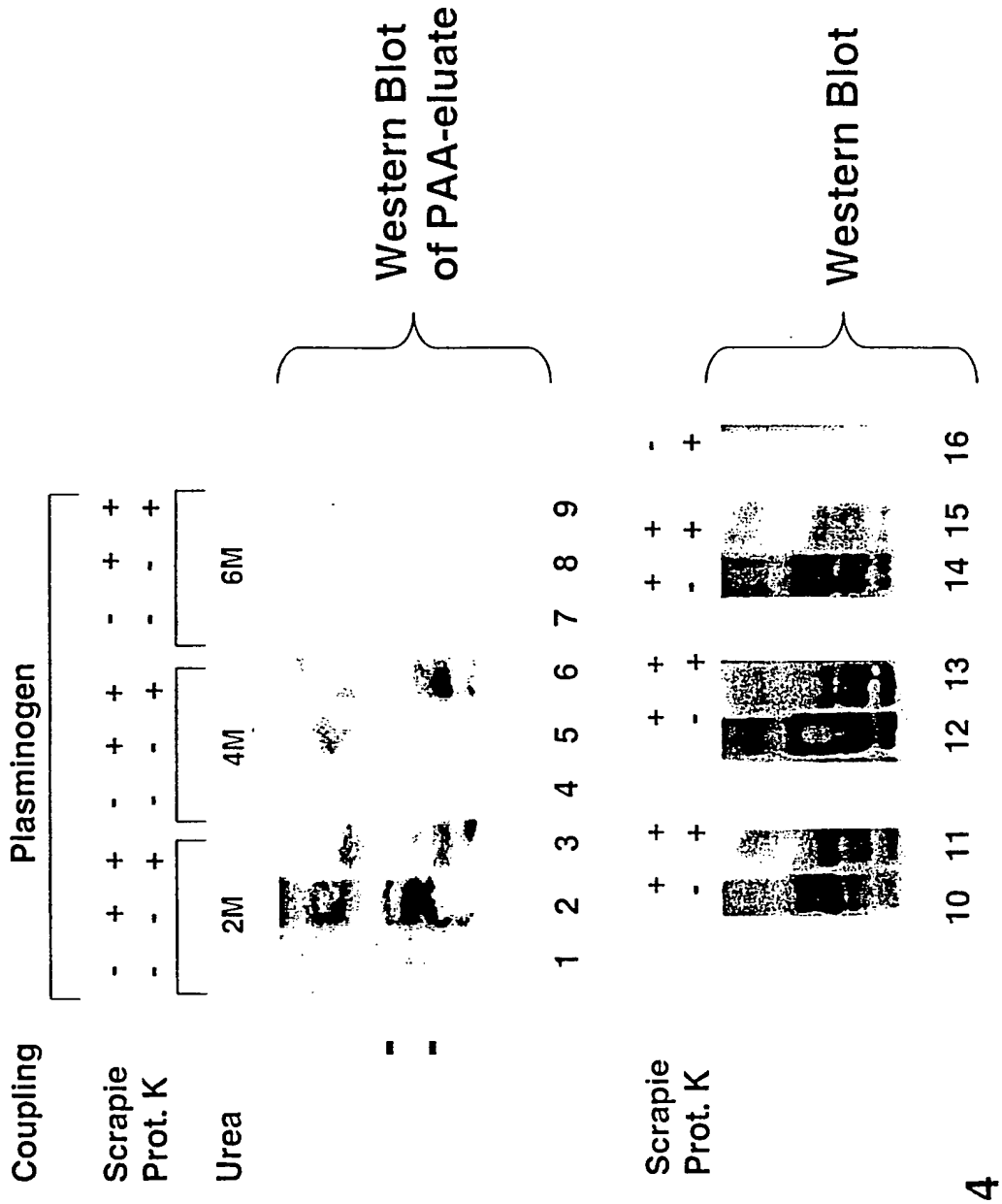

Western Blot of PAA-eluate
Western Blot
Coupling
Plasminogen
Scrapie Prot. K
Urea
2M
4M
6M
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16

Fig. 14

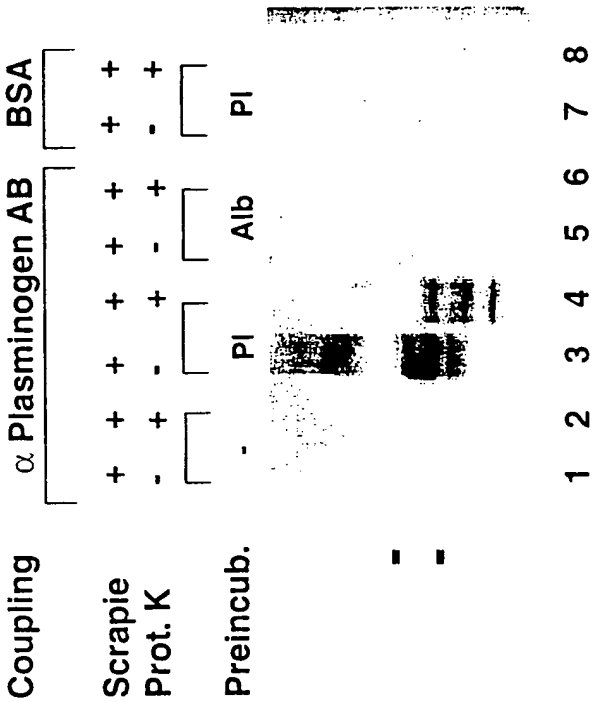

Coupling
α Plasminogen AB
BSA
Scrapie
Prot. K
Preincub.
PI
Alb
PI
1 2 3 4 5 6 7 8

Fig. 15

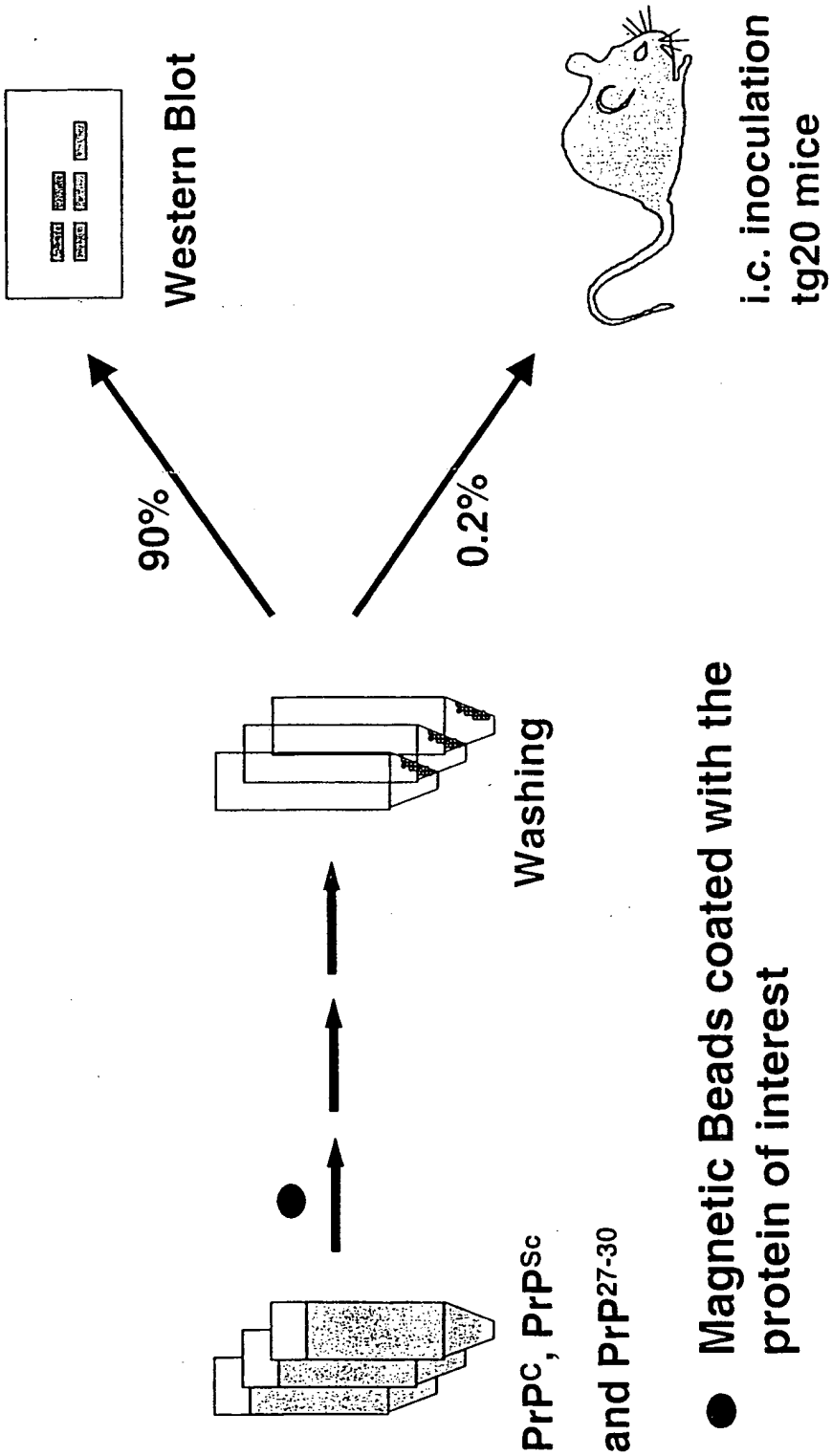
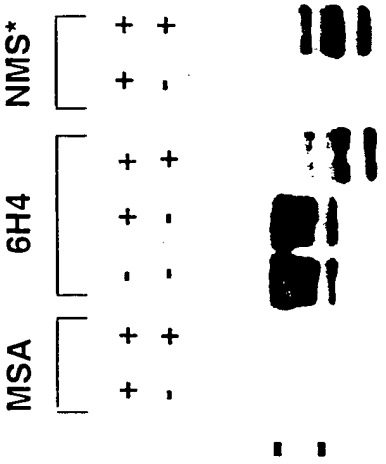

Western Blot
i.c. inoculation of tg20 mice
90%
0.2%
Washing
PrP^C, PrP^Sc and PrP^27-30
Magnetic Beads coated with the protein of interest

Fig. 16



	1	2	3	4	5	6	7
Incubation Time (d)	>110	>110	>110	82 89	71 76	>110	72 82
	>110	>110	>110	91 93	76 84	>110	82 87
	>110	>110	>110	93	84	>110	91
	>110	>110	>110			>110	
	>110	>110	>110			>110	
Mean (d)	>110	>110	>110	89.6	78.2	>110	82.8
STDV (d)				4.6	5.7		7.1
log LD_{50} (on total beads)	<2.9	<2.9	<2.9	4.7	5.7	<2.9	5.3

Fig. 17

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/IB 00/00849

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 846 533 A (BURTON DENNIS R ET AL) 8 December 1998 (1998-12-08) column 9, line 61, claims	1-11, 15-30, 33-36
X	WO 97 43649 A (WINNACKER ERNST LUDWIG ;WEISS STEFAN (DE); EDENHOFER FRANK (DE); R) 20 November 1997 (1997-11-20) claims, examples, page 6, second part "bound to solid phase"	1-11, 15-30, 33-36
P,X	WO 00 26238 A (IMP COLLEGE INNOVATIONS LTD ;JACKSON GRAHAM STUART (GB); COLLINGE) 11 May 2000 (2000-05-11) examples, esp. page 68, item 23, item 7, page 32ff	1-30, 33-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2000	10.10.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kronester-Frei, A

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/IB 00/00849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	LIU, AIZHUO ET AL: "NMR experiments for resonance assignments of 13C, 15N doubly-labeled flexible polypeptides: Application to the human prion protein hPrP(23-230" JOURNAL OF BIOMOLECULAR NMR, (FEB., 2000) VOL. 16, NO. 2, PP. 127-138. , XP000918003 whole document, esp. Methods on page 128ff	1-30, 33-36
P,Y	SWIETNICKI W ET AL: "Aggregation and fibrillization of the recombinant human prion protein huPrP90 -231." BIOCHEMISTRY, (2000 JAN 18) 39 (2) 424-31. , XP000887071 Abstract, page 425, left column	1-30, 33-36
E	WO 00 43782 A (SAFAR JIRI G ;UNIV CALIFORNIA (US); PRUSINER STANLEY B (US)) 27 July 2000 (2000-07-27) whole document, esp. page 22	1-30, 33-36
Y	JACKSON G S ET AL: "REVERSIBLE CONVERSION OF MONOMERIC HUMAN PRION PROTEIN BETWEEN NATIVE AND FIBRILOGENIC CONFORMATIONS" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 283, no. 283, 1999, pages 1935-1937, XP000887243 ISSN: 0036-8075 whole document, esp. page 1935 right column, Fig 3, digestion with PK	1-30, 33-36
Y	SHAKED G M ET AL: "Protease-resistant and detergent-insoluble prion protein is not necessarily associated with prion infectivity." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 JUN 18) 274 (25) 17981-6. , XP000919442 Abstract	1-30, 33-36
Y	AUCOUTURIER, PIERRE (1) ET AL: "Biochemical and conformational variability of human prion strains in sporadic Creutzfeldt-Jakob disease." SOCIETY FOR NEUROSCIENCE ABSTRACTS., (1999) VOL. 25, NO. 1-2, PP. 1854. MEETING INFO.: 29TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE. MIAMI BEACH, FLORIDA, USA OCTOBER 23-28, 1999 SOCIETY FOR NEUROSCIENCE. , XP000917988 Abstract, Fig. 1	1-30, 33-36

-/--

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IB 00/00849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	RISOEN P A ET AL: "One-step magnetic purification of recombinant DNA-binding proteins using magnetizable phosphocellulose." PROTEIN EXPRESSION AND PURIFICATION, (1995 JUN) 6 (3) 272-7. , XP000917983 whole document, esp. page 273, right column	1-30, 33-36
Y	EP 0 332 022 A (BEHRINGWERKE AG) 13 September 1989 (1989-09-13) claims, page 4, lines 25ff	1-30, 33-36
A	EP 0 861 900 A (ZUERICH ERZIEHUNGSDIREKTION) 2 September 1998 (1998-09-02) claims, examples	1-30, 33-36
A	SCHREUDER B E C ET AL: "PRECLINICAL TEST FOR PRION DISEASES" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 381, 13 June 1996 (1996-06-13), page 563 XP000579899 ISSN: 0028-0836 whole document	1-30, 33-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 00/00849

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 31,32
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 24, 30 and 36 are directed to a diagnostic method practised on the human/animal body, although in claims 1-21 the possibiliy of an in vivo method has not been explicitly excluded, the search has been carried out and based on the alleged effects of the compound/composition.
Although claim 34 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 31,32

Rule 39.1(ii) PCT - Transgenic animal or knockout animal (animal variety) per se used in bioassay

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. onal Application No
PCT/IB 00/00849

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5846533 A	08-12-1998	AU 707484 B	08-07-1999
		AU 7073596 A	01-04-1997
		CA 2231409 A	20-03-1997
		EP 0852011 A	08-07-1998
		JP 2000500005 T	11-01-2000
		NZ 318689 A	25-02-1999
		WO 9710505 A	20-03-1997
WO 9743649 A	20-11-1997	EP 0914614 A	12-05-1999
WO 0026238 A	11-05-2000	AU 6480799 A	22-05-2000
WO 0043782 A	27-07-2000	NONE	
EP 0332022 A	13-09-1989	DE 3807904 A	21-09-1989
		AT 114667 T	15-12-1994
		AU 3113589 A	14-09-1989
		CA 1339199 A	05-08-1997
		DE 58908667 D	12-01-1995
		DK 114689 A	11-09-1989
		ES 2064373 T	01-02-1995
		FI 891098 A	11-09-1989
		HR 940756 A	30-06-1997
		JP 2152997 A	12-06-1990
		JP 2756297 B	25-05-1998
		PT 89963 A,B	10-11-1989
		US 5672687 A	30-09-1997
		YU 49189 A	31-08-1991
EP 0861900 A	02-09-1998	WO 9837210 A	27-08-1998
		AU 6498698 A	09-09-1998
		EP 1007700 A	14-06-2000